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Behaviour of *Escherichia coli* strains along the semi-hard and hard raw milk cheese production process

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ABSTRACT

The behaviour of two *Escherichia coli* strains, including a strain with a high and a strain with a low thermotolerance phenotype, was investigated during production and ripening of Swiss semi-hard and hard raw milk cheese. In semi-hard cheese, counts of *E. coli* increased during production, before a log-linear decrease occurred during ripening with faster reduction rate in core than in rind samples, and faster reduction of the more heat sensitive strain in rind samples. Nevertheless, at the end of semi-hard cheese ripening, *E. coli* were present at least at 1.3 $\log_{10}$ cfu g^{-1} in rind samples and remained detectable by enrichment of core samples. During the first day of hard cheese production, both *E. coli* strains were almost completely inactivated. Detection by enrichment was possible in one of twelve spiked cheeses after 16 weeks, indicating the potential of a thermotolerant *E. coli* strain to survive until the end of ripening.

1. Introduction

For the production of Swiss cheese, raw milk is commonly used, as the milk enzymes add to the desired taste of the cheese. Raw milk, however, might be contaminated during the milking process with faecal bacteria, of which *E. coli* is a representative. During the cheese-making process, such *E. coli* strains encounter different stress factors, including cooking, low water activity and acidification of the cheese, which together may influence growth and survival and thus demand adaptation of the bacteria to such conditions (Peng, Tasara, Hummerjohann, & Stephan, 2011). Therefore, the corresponding stress response mechanisms are thought to be important in surviving the cheese-production process.

Recently, a collection of *E. coli* strains, which were previously isolated from raw milk cheese, were characterized based on their behaviour in view of different stresses (sub-pasteurisation temperatures, pH, salt concentration; Peng, Stephan, Hummerjohann, Blanco, & Zweifel, 2012), and a selection thereof was used for challenge studies in semi-hard model cheese (Peng et al., 2013). It was shown that two generic *E. coli* strains survived similar or better than three Shiga toxin-producing *E. coli* (STEC) strains. Due to biosafety restrictions changes to typical Swiss raw milk cheese recipe were required, including size and form of the cheese and the application of wax coating instead of the typical red smear, which alters carbon dioxide (CO_2) diffusion and pH changes during ripening.

In order to perform challenge tests in cheese that meet the conditions in commercial production of typical Swiss semi-hard and hard raw milk cheese, two generic *E. coli* strains, of which one exhibits an increased heat resistance (Peng, Hummerjohann, Stephan, & Hammer, accepted for publication), were selected for this study. The objectives of this study were (1) to investigate the fate of the two *E. coli* strains during production and ripening of the semi-hard and hard raw milk cheese, (2) to compare differences in inactivation between these two strains, and (3) to compare inactivation in the core and in the outermost edible part 5 mm below the rind of semi-hard raw milk cheese.

2. Materials and methods

2.1. Escherichia coli strains

The strains FAM21843 of serotype O178:H12 and K303 of serotype O9:H21 were previously isolated from raw milk cheese and were further characterized based on phenotypic traits in respect of cheese-related stress response (Peng et al., 2012). Strain FAM21843 exhibits an increased resistance to heat (D_{65} of 27.1 ± 6.5, Peng et al., accepted for publication) and harbours the *clpK* gene associated with the thermotolerance phenotype (Bojer, Struve, Ingmer, Hansen, & Krogfelt, 2010) in contrast to strain K303, which lacks this gene and is more sensitive to heat (D_{65} of 2.9 ± 0.3). Strain FAM21843 is trimethoprim-resistant and able to ferment adonitol, while strain K303 lacks these properties, which were used for selective detection and enumeration of the strains.

2.2. Preparation of Escherichia coli spiking cultures

After growing separately in modified tryptone soya broth (mTSB, Oxoid, Pratteln, Switzerland), the *E. coli* strains were milk-adapted by subculturing them twice in sterile skim milk, before they were diluted to inoculate the final spiking cultures in sterile milk depending on the target concentration in the vat milk. For each cheese vat, a 100 mL spiking culture was prepared and kept refrigerated until inoculation.

2.3. Preparation of starter cultures

For the production of semi-hard cheeses, two 50 mL starter cultures, MK401 and MK150, were used (raw mixed cultures of *Lactobacillus delbrueckii* ssp. *lactis, Streptococcus thermophilus*, and *Lactococcus lactis* ssp. *lactis*, Agroscope Liebefeld-Posieux), which were incubated in sterile skim milk at 38 °C for 15 h (MK150) and 6 h (MK401), respectively, before using for cheese making.

For the production of hard cheeses, two 120 mL starter cultures, MK101 and MK124, were used (raw mixed cultures of *Lactobacillus delbrueckii* ssp. *lactis* and *Streptococcus thermophilus*, Agroscope Liebefeld-Posieux), which were incubated at 38 °C for 15 h (MK124) and 6 h (MK101) before use for cheese making, respectively.

2.4. Production of spiked semi-hard raw milk cheese

After inoculation of 80 L warmed raw milk (32 °C) with the spiking culture and the starter cultures an Appenzeller-type cheese was produced. Rennet (Winkler GR orange, Winkler AG, Konolfingen, Switzerland) was added 30 min after the starter cultures to induce coagulation within 35 min. The curd was cut into 0.4-0.8 cm cubes, cooked at 46 °C for 15 min and subsequently filled into a circular form (diameter 30 cm). The loaf was put into a pressing chamber operating a temperature programme (34 °C for 4 h, 32 °C for 4 h, 28 °C for 8 h, 26 °C for 4 h). After 20 h in the pressing chamber, the cheese was put into 22 % (wt/vol) brine at 12 ± 1 °C for 1 d. Subsequently, the cheese was ripened at 14-15 °C and a relative humidity of 90-96% for 16 weeks. For each spiking level, four cheeses were produced containing both *E. coli* strains and an additional four cheeses were produced without spiking as negative controls, resulting in a total of 12 semi-hard cheeses.

2.5. Production of spiked hard raw milk cheese

After inoculation of 120 L warmed raw milk (32 °C) with the spiking culture and the starter cultures a Gruyère-type cheese was produced. Rennet (Winkler GR orange) was added 30 min after the starter cultures to induce coagulation within 40 min. The curd was cut into 0.3-0.6 cm cubes, cooked at 53 °C for 20 min and subsequently filled into a circular form (diameter 35 cm). The cheese loaf was put into a pressing chamber operating a temperature programme (cooling from 53 °C to 40 °C within 2 h, to 35 °C within 2 h, to 32 °C within 4 h and to 25 °C within 12 h). After pressing, the cheese loaf was placed in 22 % (wt/vol) brine at 12 ± 1 °C

for 1 d. Subsequently, the cheese was ripened at 14-15 °C and a relative humidity of 90-96% for 16 weeks. Four cheeses were produced for each spiking combination: (1) both *E coli* strains in the same cheese, (2) only strain FAM21843, (3) only strain K303 and (4) an additional four cheeses were produced without spiking as negative controls, resulting in a total of 16 hard cheeses.

2.6. Sampling of semi-hard and hard raw milk cheese

During production, spiked raw milk was sampled before addition of the starter cultures, and the curd was sampled after filling into the cheese forms. The curd of the hard raw milk cheese was additionally sampled before cooking. During ripening of the semi-hard raw milk cheese, samples were taken 5 mm below the rind and from the core (10 cm from the outside radially, 2 cm from the outside vertically) of the cheese after 1 d, 1, 2, 4, 6, 8, 12 and 16 weeks. Hard cheese was sampled close to the rind, like the semi-hard cheese, but not in the core.

2.7. Detection of Escherichia coli

Cheese samples of 10 g were processed as described in Peng et al. (2013) for the identification and enumeration of FAM21843 on adonitol-MacConkey agar containing trimethoprim, and of K303 on adonitol-MacConkey agar, respectively. At random, isolates were further genotyped by repetitive sequence-based PCR (repPCR) according to Mohapatra, Broersma, & Mazumder (2007) using a (GTG)5 primer.

If at least one of the *E. coli* strains was not quantitatively detected, another 10 g cheese sample was taken for enrichment procedure and homogenized with 90 g mTSB (Oxoid) / acriflavin (12 mg L^{-1}, Sigma-Aldrich) using a stomacher. For detection of the *E. coli* strains, 10 µl of the enrichment broth was streaked out on adonitol-MacConkey agar and adonitol-MacConkey agar containing trimethoprim. At random, isolates were genotyped by repPCR according to the method of Mohapatra et al. (2007).

136

137 *2.8. Chemical and physical analysis of cheese*

138 Dry matter (DM), contents of fat, and sodium chloride content of the cheese samples were
139 analysed by using standard methods after ISO/IDF, pH was measured potentiometrically, total
140 lactic acid (TLA) content was determined by using enzymatic UV test kit (R-Biopharm,
141 Darmstadt, Germany) and a_w value was measured according to the Swiss Food Manual,
142 Chapter 64 (1991).

143

144 *2.9. Statistical Analysis*

145 Colony counts (CC) were log-transformed and samples below the quantitative limit of
146 detection (LOD, < 10 cfu g^{-1}) were set at a log value of 0. Data were analysed by repeated
147 measurement analysis of variance (ANOVA), ANOVA and linear regression by using IBM
148 SPSS Statistics Version 20 (IBM Corporation, Armonk, US).

149

150 **3. Results and discussion**

151 *3.1. Semi-hard raw milk cheese*

152 The chemical and physical parameters were analysed to determine if the cheese composition
153 corresponds to typical composition in practice (Table 1). The mean DM content of all cheese
154 samples taken at day 1 was 60.0% and the mean percentage of moisture on a fat-free basis
155 (MFFB) was 57.5%, which was rather low, probably due to the relatively high cooking
156 temperature of 46°C, but still in the range for a semi-hard cheese. Mean TLA content and pH
157 of day 1 cheese samples were at 140.4 mmol kg^{-1} and 5.3, respectively, which are both in the
158 expected range. During ripening, the pH values increased to 5.8 in the rind and 6.1 in the core.
159 The higher pH value in the core was not expected. Most probably, this was due to mould
160 growth between week 12 and week 16 in the holes produced when sampling the cheese in the
161 core. While the outer parts of the cheeses were not affected, the results from core samples of

week 16 were discarded. The DM content increased about 5% while ripening. At the end of ripening, i.e., after 4 months, the mean MFFB content of all cheese samples was at 52.1%. The chemical and physical parameters did not significantly differ between the cheeses made from different spiking levels and the cheeses produced without spiking. Therefore, the *E. coli* strains did not influence the ripening process.

The raw milk used for cheese production of the two cheese types was tested negative for the presence of *E. coli* by enrichment of 25 ml samples taken before addition of spiking and starter cultures (data not shown). During the manufacture, an increase in cfu mL^{-1} for *E. coli* was observed in rind and core samples (Table 2), which occurred similar to other studies and is attributed to a concentration effect and growth of the *E. coli* strains (Montet et al., 2009; Schlesser et al., 2006). The lower counts in the core at day 1 were most probably caused by the slower cooling of the curd in the centre of the cheese (Ercolini, Fusco, Blaiotta, Sarghini, & Coppola, 2005), which applied a stronger thermal stress on the *E. coli* strains. This is further supported by the observation that strain FAM21843, which exhibits an increased thermotolerance, was less reduced in the core than strain K303 ($P < 0.01$ at high spiking level).

During ripening, a continuous decrease was observed for both strains in rind and core samples (Fig. 1). The reduction of the *E. coli* strains is attributed to the sum of the stresses occurring in the cheese (Peng et al., 2011), and can be described with high accuracy by a log-linear model. In the core of the cheese, the decrease occurred significantly faster ($P < 0.001$) for both *E. coli* strains at both spiking levels than in rind samples (Table 3 and Fig. 1). While a faster reduction ($P < 0.001$) was observed for strain K303 than for strain FAM21843 in rind samples at both spiking levels, the decrease occurred at a similar rate in the core. The two *E. coli* strains were quantitatively detected in all rind samples over the whole ripening period, while the majority of core samples were below the LOD after eight weeks. At the end of ripening, *E. coli* were not directly quantifiable from core samples, but still detected by enrichment in

188 every sample. The reduction in rind samples occurred similar as the reduction observed by
189 Peng et al. (2013) in smaller cheeses by taking bore samples (without differentiation between
190 rind and core zone).
191 Ross, Zhang, & McQuestin (2008) proposed that the rate of inactivation under growth-
192 preventing conditions depends more on temperature than on pH or water activity (a_w), and so
193 a further decrease of pH or a_w would not considerably accelerate the inactivation at already
194 growth-preventing conditions. Therefore, the differences in reduction of the *E. coli* strains
195 between core and rind zones are most probably not caused by the small differences in water
196 activity or pH. Since the ripening chamber was held at constant temperature, the faster
197 reduction of *E. coli* in the core of the cheese was also not caused by a variation in
198 temperature. Another stress, which is encountered stronger in the core of the cheese and may
199 contribute to the faster decrease of *E. coli* is the higher partial pressure of carbon dioxide
200 (CO_2) in the core, exhibiting an inhibitory effect on *E. coli* (Dixon & Kell, 1989). In cheese,
201 CO_2 is produced by lactic acid bacteria (LAB) and diffuses from the centre to the outside,
202 which results in a considerably higher partial pressure of CO_2 in the core of the cheese (Blanc,
203 Bosset, & Pauchard, 1980; Pauchard, Flueckiger, Bosset, & Blanc 1980). In addition, the
204 deacidification of smeared cheeses typically occurs faster close to the rind than in the core.
205 Therefore, the outermost edible part of a cheese potentially constitutes the most favourable
206 environment for survival of *E. coli*.
207
208 *3.2. Hard raw milk cheese*
209 Also for the hard cheese, the chemical and physical parameters were analysed to determine if
210 the cheese composition corresponds to typical composition in practice (Table 1). On an
211 average, pH increased from 5.3 to 5.6 and a_w decreased from 0.973 at day 7 to 0.941 after 16
212 weeks. At the end of ripening, DM, pH, a_w, moisture on a fat-free bases, TLA content and pH
213 were all in the expected range and close to values in four months old Swiss Gruyère cheese.

214 Physicochemical parameters were not significantly different between cheeses made from
215 different spiking variants and cheeses produced without spiking.
216 The two *E. coli* strains exhibited a similar increase of about 1 $\log_{10}$ cfu g^{-1} from raw milk to
217 the curd before cooking (Table 4), which is attributed to a physical concentration effect.
218 Thereafter, strain FAM21843 was reduced by about 0.5 $\log_{10}$ cfu g^{-1} during cooking of the
219 curd, while strain K303 was almost completely inactivated and only detected in one of the
220 eight curd samples after cooking. Thereafter, strain K303 was detected by enrichment in two
221 out of eight samples taken after 4 weeks. The cooking temperature of a hard raw milk cheese
222 is considerably higher than for a semi-hard cheese, and this stronger reduction was expected,
223 especially for strain K303, which lacks increased thermotolerance. Nevertheless, the
224 thermotolerant strain FAM21843 was also no longer countable by direct plating (LOD at < 10
225 cfu g^{-1}) in any cheese sample at day 1 or thereafter. After the curd was filled into the forms,
226 the *E. coli* strains still encountered an increased temperature and additional stresses as
227 acidification and pressing of the cheese, which inactivated also strain FAM21843 almost
228 completely within the first day of cheese production. However, the detection by enrichment of
229 strain FAM21843 was possible during ripening in 16 of 32 cheese samples taken before week
230 4 and thereafter in two samples, which were taken after 8 and 16 weeks, respectively. At the
231 end of ripening, FAM21843 was detected after enrichment in one of the eight cheeses initially
232 spiked with this strain.
233
234 **4. Conclusion**
235 Two different cheese types were spiked with two different *E. coli* strains to investigate their
236 fate during production and ripening of the cheese. In hard raw milk cheese, both *E. coli*
237 strains, including a thermotolerant strain, were almost completely inactivated within the first
238 day of cheese production. During ripening, the detection of the thermotolerant *E. coli* strains
239 was possible in several samples, including one at the end of the ripening period. With regard

to the spiking level and the conditions used during cheese production, which were favourable for survival of *E. coli,* the potential of *E. coli* surviving until the end of ripening in Swiss hard raw milk cheese is expected to be low.

In semi-hard cheese, both *E. coli* strains were quantified at least at 1.3 $\log_{10}$ cfu g^{-1} close to the rind and remained detectable by enrichment in core samples at the end of the ripening period. These observations are in accordance with the model cheese study (Peng et al., 2013) and show that *E. coli* are able to survive a typical commercial production process of a Swiss semi-hard raw milk cheese. In addition, a difference in *E. coli* counts was observed between rind and core samples, which implies that differences in encountered stresses occur within the cheese and therefore shape and size of the cheese can be important for survival of *E. coli*. These observations should be taken into consideration in view of food safety, as the outermost edible part probably offers the most favourable conditions for survival of *E. coli*.

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305 **Table 1**

306 Physical and chemical parameters of the semi-hard and hard raw milk cheese at the end of

307 ripening. Mean values and standard deviations are shown.

	Semi-hard raw milk cheese (n = 24)	Hard raw milk cheese (n = 16)
Dry matter (%)	65.1 ± 1.6	67.2 ± 0.3
Fat in dry matter (%)	50.7 ± 1.0	51.0 ± 0.5
Moisture on a fat-free basis (%)	52.1 ± 2.0	50.0 ± 0.5
Sodium chloride (%)	1.8 ± 0.2	1.6 ± 0.1
Total lactic acid (mmol kg^{-1})	72.6 ± 6.4[a]	106.9 ± 7.4

308 [a] Only rind samples

309 **Table 2**

310 Fate of two *E. coli* strains within the first day of semi-hard raw milk cheese production. Mean
311 value and standard deviations are shown for samples taken from four cheeses made at the
312 same conditions (log_{10} cfu g^{-1}).

Strain	Spiking level	Raw milk	Curd	Rind (1 d)	Core (1 d)
FAM21843	Low	2.1 ± 0.1	3.9 ± 0.0	5.2 ± 0.1	4.7 ± 0.5
	High	3.9 ± 0.0	5.6 ± 0.2	7.0 ± 0.3	6.4 ± 0.4
K303	Low	1.7 ± 0.2	3.8 ± 0.0	5.0 ± 0.1	4.0 ± 0.5
	High	3.8 ± 0.1	5.5 ± 0.2	6.6 ± 0.4	5.0 ± 0.4

313 **Table 3**

314 Reduction rates of two *E. coli* strains during the 16-week ripening period of semi-hard raw
315 milk cheese. Mean values, standard deviations and coefficients of determination (R^2) are
316 shown for samples taken from four cheeses made at the same conditions (reduction per week
317 in $\log_{10}$ cfu g^{-1}).

	Low spiking level		High spiking level	
	FAM21843	K303	FAM21843	K303
Rind	0.13 ± 0.01	0.24 ± 0.01	0.18 ± 0.01	0.27 ± 0.02
R^2	0.82	0.92	0.93	0.89
Core	0.43 ± 0.04	0.40 ± 0.06	0.50 ± 0.07	0.56 ± 0.07
R^2	0.80	0.69	0.70	0.79

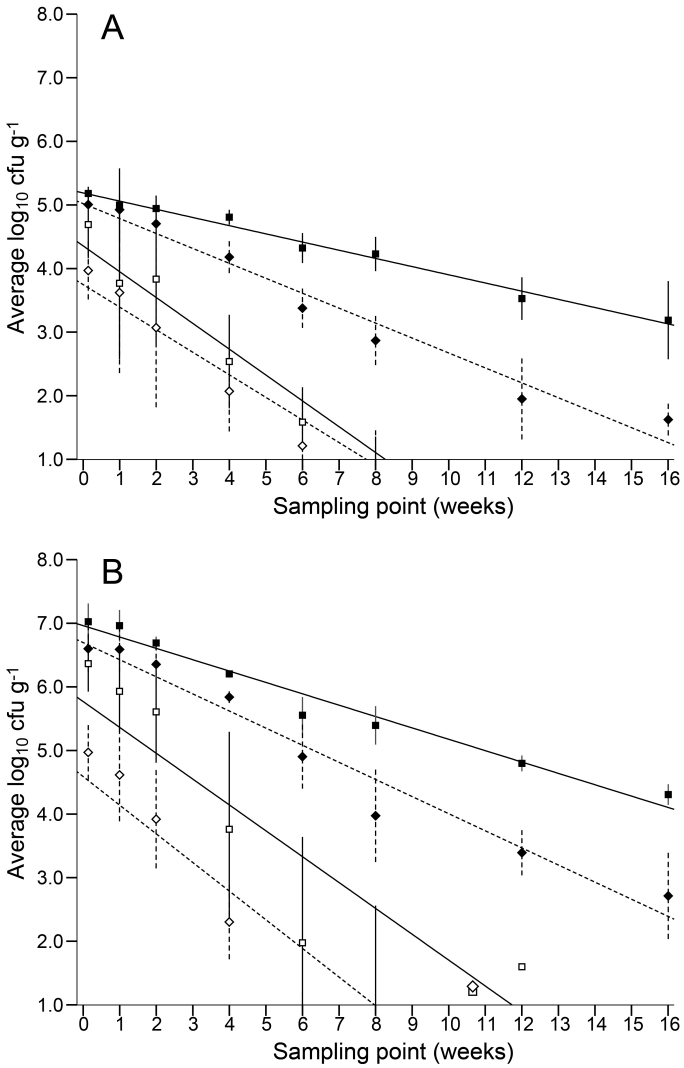
318 **Table 4**

319 Fate of two *E. coli* strains during production of hard raw milk cheese. Mean value and
320 standard deviations are shown for samples taken from eight cheeses made at the same
321 conditions (log_{10} cfu g^{-1}).

Strain	Raw milk	Curd before cooking	Curd after cooking	Rind (day 1)
FAM21843	3.7 ± 0.1	4.7 ± 0.2	4.2 ± 0.6	< 1.0[a]
K303	3.2 ± 0.2	4.5 ± 0.2	< 1.0[a]	< 1.0[a]

322 [a] Below limit of quantitative detection.

323


A
B
Average log10 cfu g-1
Sampling point (weeks)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

324

325
326 **Fig. 1.** Behaviour of the *Escherichia coli* strains FAM21843 (■, solid line) and K303 (◆,
327 dashed line) during ripening of Swiss semi-hard raw milk cheese in rind (filled symbol) and
328 core samples (empty symbols) after low (A) and high spiking (B). Mean values, standard
329 deviations and regression lines of samples taken from four cheeses made at the same
330 conditions are shown.